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16. ABSTRACT

Only a small percentage of the heterotrophic bacteria encountered in water distribution systems are identifiable because many of these organisms fail to grow on the conventional media used for biochemical characterization. Organisms that would not subculture from the same standard plate count agar used for initial isolation were successfully subcultured on a low-nutrient medium, R3A. These cultures were then inoculated to a modified O/F base medium containing specific substrates. This, combined with a lower incubation temperature (30°C), increased the enzymatic activity of many of the organisms. These reactions established a groundwork for tentative taxonomy.

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Characterization of Dysgonic, Heterotrophic Bacteria from Drinking Water

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Only a small percentage of the heterotrophic bacteria encountered in water distribution systems are identifiable, because many of these organisms fail to grow on the conventional media used for biochemical characterization. Organisms that would not subculture from the same standard plate count agar used for initial isolation were sucessfully subcultured on a low-nutrient medium, R3A. These cultures were then inoculated to a modified O/F base medium containing specific substrates. This, combined with a lower incubation temperature (30°C), increased the enzymatic activity of many of the organisms. These reactions established a groundwork for tentative taxonomy.

The heterotrophic plate count has often been used as a supplemental indicator of water treatment effectiveness and of the general microbial quality of drinking water. Because the majority of bacteria isolated from the heterotrophic bacterial population fail to grow when subcultured to a conventional medium (9), they cannot be identified. The purpose of this investigation was to biochemically characterize representative, nonclassifiable, heterotrophic gram-negative bacteria isolated from a municipal water supply. After these organisms were initially isolated on standard plate count agar (SPCA) (1), the use of R3A medium (9) allowed for their continued propagation. Hugh and Leifson (6) developed a base medium (O/F) that, when augmented with various substrates and at a lower incubation temperature (30°C), permitted the biochemical characterization of these dysgonic (scant-growing) organisms.

MATERIALS AND METHODS

Selection of isolates. A total of 100 bacterial isolates from 10 samples collected from a municipal water distribution system were studied. The water samples contained no residual chlorine. All of these bacterial isolates were initially recovered on SPCA. Colonies from the SPCA were subcultured to fresh SPCA plates for purification and also to a new medium, R3A, used for subculturing dysgonic bacteria from potable water. Almost 85% of the subcultures on the fresh SPCA plates failed to grow; this necessitated picking subcultured colonies of each isolate from the R3A plates to R3A slants for further analyses. Subcultures from the R3A slants were inoculated into O/F-glucose medium, in pairs, contained in borosilicate glass to avoid alkali leaching and pH alterations. After 24 to 48 h of incubation at 35°C, all tubes were observed for fermentation of glucose, or oxidation of glucose, or no reaction (in which case the reaction mixture remained green).

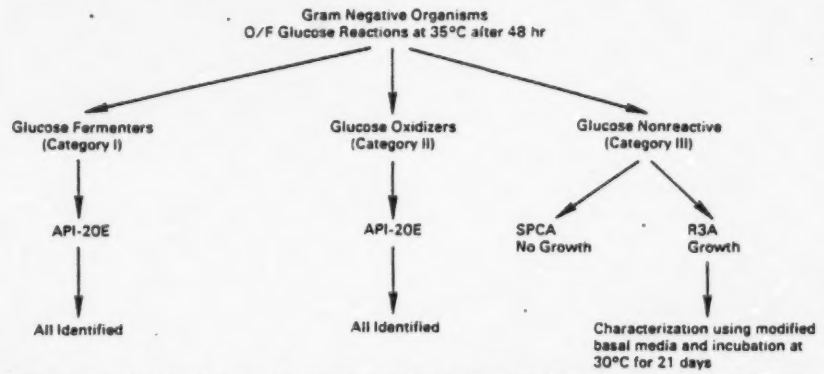

FIG. 1. Flow diagram for identification and characterization of gram-negative isolates from drinking water systems.

On the basis of the reaction of the selected isolates to O/F medium, the isolates were divided into three categories (Fig. 1): category 1 (category I in figure), glucose fermenters; category 2 (category II in figure), glucose oxidizers; and category 3 (category III in figure), glucose nonoxidizers. Organisms in categories 1 and 2 were again cultured with the use of conventional procedures (API-20E system; Analytab Products, Plainview, N.Y.) and identified.

The organisms in category 3, subculturable only to R3A, were not identifiable by conventional methods (i.e., no

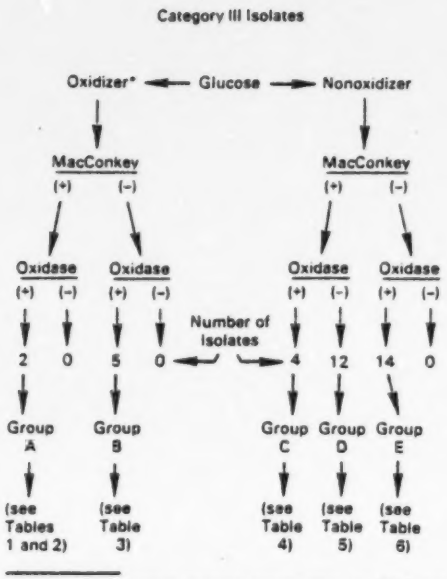

FIG. 2. Preliminary characterization of category 3 isolates based on glucose, growth on MacConkey, and oxidase reactions from the Weaver schema (12).

observable growth on API-20E systems or most conventional media). Cultures in category 3 were then inoculated a second time into single O/F-glucose medium tubes, with no oil overlay, and incubated this time at 30°C instead of 35°C for up to 21 days. Oxidase tests were done for isolates from the R3A medium. MacConkey agar slants were inoculated for observation of growth or no growth after the procedure for identification of nonfermenters (12). Category 3 isolates were further characterized by reactions on modified O/F medium with specific substrates and incubated at 30°C.

Media and methods. The base O/F medium (6), which was modified for category 3 isolates, contained 2.0 g of tryptone, 5.0 g of NaCl, 0.3 g of K_2HPO_4, 1.0 to 15.0 g of agar depending on the specific substrate, and 1,000 ml of water. To determine carboxylation and dihydrolation, three individual amino acids (to a concentration of 5 g/liter) were added, each to an individual flask, to the modified O/F medium: L-ornithine, L-arginine, and L-lysine. A fourth flask, the control, contained no amino acid substrate. In addition, 0.02 g of bromocresol purple, 1.0 g of glucose, and 2.0 g of agar were added to each flask. The pH was adjusted to 6.5, and the medium was dispensed into tubes (13 by 100 mm), which were then capped with conventional caps and autoclaved. After inoculation of the medium with category 3 isolates, the caps were closed tightly to exclude air, and the tubes were examined daily and compared with the substrate controls containing no amino acids. Because glucose-nonfermenting organisms do not give acid reactions under these conditions, the tubes were examined for dark-purple alkaline reactions. Use of the modified O/F medium also made possible the detection of indole production from tryptone.

Nitrate, nitrite, tetrazolium, selenium, and methylene blue reactions were tested as previously described (2, 4, 8), except that the modified O/F medium was used as the base medium. The following amount of agar was added to the base medium in each test: nitrate and nitrite, 0.1%; tetrazolium and selenium, 0.3%; methylene blue, 0.2%. The methylene blue medium also contained 0.1% glucose. The pH was adjusted to between 6.8 and 7.2. Permeable-membrane caps (Kimball Glass, Owens, Illinois, Inc., Toledo, Ohio) were used on tubes for the nitrate, nitrite, and methylene blue reduction reactions. Streak plates were used for the tetrazolium and selenium reduction reactions to facilitate the detection of color changes.

TABLE 1. Characterization of glucose-oxidizing, MacConkey-positive, oxidase-positive water distribution system isolates, group A, by O/F tubes

Weaver test	Reaction[a] at 30°C[b] for isolate:	
	60	62
Glucose	A/N	A/N
Xylose	B	B
Mannitol	A	B
Lactose	B	B
Sucrose	B	A/N
Maltose	B	A

[a] N, Neutral; A, acid production; B, basic reaction; A/N, acid top, neutral butt.
[b] At 35°C, isolates 60 and 62 were nonreactive.

TABLE 2. Reactions of glucose-oxidizing, MacConkey-positive, oxidase-positive water distribution system isolates, group A

Test	Reaction[a] on base medium[b] of isolate:	
	60	62
Weaver (12)		
Indole	−	−
Gas from nitrate	+	−
Nitrite formation	+	−
Complete reduction of nitrate and nitrite	+	+
SS agar (for growth)[c]	NG[d]	NG
Lysine decarboxylase	−	+
Arginine dihydrolase	+	−
Motility	G[d]	G
Complementary reactions		
Reduction		
Tetrazolium	−	−
Methylene blue	+	−
Hydrolysis		
Selenium	−	−
Urea	+	+
Starch	−	+
Esculin	−	+
Hippurate	+	−
Gelatin	−	−
Amino acid		
Ornithine	−	−
Arginine	+	−
Lysine	−	+
Other (DNase)	−	−

[a] +, Positive reaction; −, negative reaction.
[b] Isolates 60 and 62 did not grow on conventional media.
[c] Conventional media were used. SS, Salmonella-shigella.
[d] NG, No growth; G, growth (nonmotile).

Starch (wheat), hippurate, gelatin, and esculin reactions were tested as previously described (2, 4, 8) by using the modified O/F base medium. The amount of agar used in each base medium was as follows: starch and esculin, 1.5%; hippurate, 0.25%; gelatin, 0.1%. The gelatin medium also contained 0.1% glucose. The pH was adjusted to between 6.8 and 7.2. Streak plates were used for starch and hippurate tests, and the semisolid hippurate plates were incubated upright. Tubes with permeable-membrane caps were used for gelatin and esculin.

To detect urease reactions, a solution of 2.0 g of urea plus 0.012 g of phenol red in 10 ml of distilled water was prepared and filter sterilized through a membrane (pore size, 0.2 μm). A 90-ml portion of the O/F base medium was prepared with 1.5 g of agar; the pH was adjusted to 6.9. After autoclaving, the medium was cooled to between 50 and 55°C, and 10 ml of sterile urea was added aseptically; the medium was then distributed into slants in sterile tubes (13 by 100 mm) with permeable-membrane caps.

The medium for testing motility reactions was prepared by combining 10.9 g of tryptone, 5.0 g of NaCl, 3.0 g of agar, 0.05 g of 2,3,5-triphenyltetrazolium chloride, 0.20 g of $FeNH_4(SO_4)_2$, 0.2 g of sodium thiosulfate, and 1,000 ml of distilled water. The pH was adjusted to 7.0, and the medium was distributed into tubes (13 by 100 mm) with permeable-medium caps.

For the DNase test, commercial medium was used as a base. The medium was prepared by combining 42.0 g of DNase test agar, 0.5 g of methyl green, and 1,000 ml of water. The pH was adjusted to 7.5. Reactive positive and negative bacterial controls were used for each substrate tested.

O/F tubes, triple sugar-iron-agar slants, salmonella-shigella plates, blood agar plates containing 5% defibrinated sheep blood, eosin methylene blue agar plates, and MacConkey agar slants were prepared as recommended by Difco Laboratories, Detroit, Mich. The defibrinated sheep blood

TABLE 3. Characterization of gram-negative glucose-oxidizing, MacConkey-negative, oxidase-positive water distribution system isolates, group B

Test	Reactions[a] in base medium at 30°C of isolate:				
	2	4	8	20	48
Weaver (12)					
Indole	−	−	−	−	−
Insoluble pigment (R3A)	Y	Y	Y	Y	Y
Glucose	A	A/N	A/N	A	A
Xylose	A/B	A/N	A/N	A	A
Mannitol	A/B	B	B	B	B
Lactose	B	B	B	A	N
Sucrose	B	B	B	B	A
Maltose	A	B	B	A	N
Urea	−	−	−	+	−
Motility	NM	NM	NM	NM	NM
Complementary reactions					
Reduction					
Nitrate	+	+	−	+	−
Nitrite	−	−	−	−	−
Tetrazolium	+	+	NG	NG	+
Methylene blue	+	+	+	−	−
Selenium	+	+	+	+	+
Hydrolysis					
Urea	−	−	−	+	−
Starch	−	−	−	+	+
Hippurate	+	+	+	−	+
Gelatin	−	−	−	−	+
Esculin	+	+	+	−	+
Amino acid					
Ornithine	+	+	−	+	+
Arginine	+	+	−	+	+
Lysine	−	−	−	+	−
Other					
Indole	−	−	−	−	−
DNase[b]	+	−	−	+	+
Motility[b]	−	−	−	−	−

[a] Y, Yellow; A, acid production; N, neutral (nonreactive); B, basic reaction; NM, nonmotile; NG, no growth.
[b] Conventional media were used.

TABLE 4. Characterization of gram-negative glucose-nonoxidizing, MacConkey-positive, oxidase-positive water distribution system isolates, group C

Test	Reactions[a] in base medium at 30°C of isolate:			
	3	12	40	45
Weaver (12)				
Flagella	1–2 polar[b]	1–2 polar	1–2 polar	1–2 polar
Nitrite reduction	−	+	+	+
Gas from nitrate	−	−	−	−
Gas from nitrite, nitrate not reduced	−	−	−	−
H_2S, butt of TSI[c]	−	−	−	−
O/F-Xylose	B	B	B	B
Growth on SS[c]	−	−	−	−
Urea	−	−	−	−
Complementary reactions				
Reduction				
Nitrate	−	+	+	+
Nitrite	−	−	−	−
Tetrazolium	+	+	−	+
Methylene blue	+	+	+	+
Selenium	+	+	+	+
Hydrolysis				
Urea	−	−	−	−
Starch	−	−	−	−
Hippurate	+	+	+	+
Gelatin	−	−	−	−
Esculin	−	−	−	+
Amino acids				
Ornithine	−	−	−	+
Arginine	+	−	−	+
Lysine	−	+	−	+
Other				
Indole	−	−	−	−
DNase[c]	−	−	−	−
Motility[c]	+	+	+	+

[a] +, Positive reaction; −, negative reaction; B, basic reaction.
[b] One or two flagella.
[c] Conventional media were used. SS, Salmonella-shigella.

TABLE 5. Characterization of gram-negative glucose-nonoxidizing, MacConkey-positive, oxidase-negative water distribution system isolates, group D

Test	Reactions[a] in base medium at 30°C of isolate:											
	10	11	15	22[b]	23[b]	24	28	33	35	39	59	61
Weaver (12)												
Urea	−	−	NG	+	−	−	−	−	−	+	−	−
Soluble pigment	O	O	O	Bu	Bu	O	O	O	O	B	Y	O
O/F-maltose	A+	−	−	−	−	A/B	−	−	−	B	A/N	−
Gelatin	−	−	+	−	−	−	−	−	NG	+	NG	−
Motility	−	−	−	−	−	−	−	−	−	−	−	−
Beta hemolysis	−	−	−	−	−	−	−	±	−	−	−	−
Complementary reactions												
Reduction												
Nitrate	−	+	−	−	+	+	−	−	−	+	+	−
Nitrite	−	−	−	−	−	+	+	−	−	−	−	−
Tetrazolium	−	−	+	+	+	+	+	+	+	+	−	−
Methylene blue	−	−	−	−	+	−	−	−	−	+	+	−
Selenium	+	+	+	+	+	+	+	−	+	+	+	−
Hydrolysis												
Urea	−	−	+	−	−	−	+	+	−	+	−	+
Starch	−	+	+	−	−	−	+	+	−	+	−	+
Hippurate	−	−	−	+	+	−	−	+	−	+	+	−
Gelatin	+	+	+	−	−	−	−	+	−	+	−	+
Esculin	+	+		−	−	+	+	+	+	+	+	+
Amino acids												
Ornithine	−	−	−	−	−	−	−	−	−	+	+	−
Arginine	−	−	−	−	−	+	−	−	−	−	+	−
Lysine	−	−	−	−	−	−	−	−	−	+	−	+
Other												
Indole	−	−	−	−	−	−	−	−	−	−	−	−
DNase[c]	+	+	+	−	−	−	+	−	−	−	+	+
Motility[c]	−	−	−	−	−	−	−	−	−	−	−	−

[a] NG, No growth; O, orange; Bu, buff; B, basic reaction; Y, yellow; A, acid production; N, neutral (nonreactive).
[b] *Acinetobacter calcoaceticus*, identified by DNA transformation test.
[c] Conventional media were used.

agar plates were prepared with R3A base medium containing 0.5% NaCl to prevent hemolysis of erythrocytes. Procedures and reagents used to detect by-products and staining techniques have been described previously (2, 4, 8, 10).

For isolates that appeared as tiny blue colonies on eosin methylene blue agar (11), a DNA transformation was performed to a competent auxotroph of *Acinetobacter calcoaceticus* (2, 7) to verify their tentative identification as *A. calcoaceticus*.

RESULTS

When the 100 isolates were subcultured to SPCA, only 15 grew; however, all 100 isolates, including the 85 that did not grow on SPCA, were successfully subcultured to R3A medium.

The 15 isolates that did grow on SPCA were readily identified by available taxonomic schema. Of the 15, 9 were identified as *Pseudomonas* spp.; the others were *Aeromonas hydrophila*, *A. calcoaceticus*, *Serratia marcescens*, *Chromobacter violaceum*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*.

Of the 85 isolates (category 3) subcultured to R3A, 48 could not be kept viable. Therefore, only the 37 that survived repeated subcultivation on R3A medium were examined further. These isolates did not grow on an API-20E system.

A growth medium was needed to which specific substrates could be added to test the metabolic activities of the different bacterial isolates. The R3A medium was not suitable, because growth in this medium resulted in the production of excess alkaline end products and the formation of a precipitate from tetrazolium. Although all the isolates could not grow on MacConkey agar, some could, and it could thus be used to separate strains by using the plan of glucose utilization described by Weaver (12), the MacConkey growth-no growth test, and the oxidase reaction. Why some isolates would not subculture to SPCA but would grow on the selective MacConkey agar is not known.

Of the numerous media tested, only O/F medium supported growth of category 3 isolates. Because the O/F medium could be easily modified to test the metabolic activity of the isolates in different substrates, it was used as the base medium for the various biochemical tests.

Although some of the isolates were able to oxidize glucose at 30°C, none carried out this reaction at 35°C (Fig. 1). Increased enzymatic activity at lower incubation temperatures has been observed previously (5). Based on this activity at a lower temperature, a taxonomic outline patterned after the one developed by Weaver (12) was developed to characterize these isolates with the use of other base

TABLE 6. Characterization of gram-negative glucose nonoxidizing, MacConkey-negative, oxidase-positive water distribution system isolates, group E

Test	Reactions[a] in base medium at 30°C of isolate:													
	1	5	6	9[b]	14	21	25	26	29	34	36	47	52	58
Weaver (12)														
Indole	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Urea	−	−	−	+	+	+	−	−	+	−	+	−	+	−
O/F-xylose	A+/N	A2+	B	A2+	N	N	N	N	N	N	N	N	N	N
Nitrate	+	+	−	−	−	−	−	−	−	−	−	−	+	−
Complementary reactions														
Reduction														
Nitrite	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tetrazolium	+	+	+	+	+	+	+	+	+	+	−	+	−	−
Methylene blue	−	+	−	+	−	−	−	+	−	−	−	−	−	−
Selenium	+	+	+	+	+	−	+	+	+	−	+	+	+	+
Hydrolysis														
Starch	+	−	−	+	+	−	+	−	+	+	+	+	+	+
Hippurate	−	+	−	−	−	−	−	+	+	−	−	−	−	−
Gelatin	−	−	+	+	−	−	−	−	−	+	+	−	+	−
Esculin	+		+	−	−		+	−	+	+	+	+	+	+
Amino acids														
Ornithine	+	−	−	+	−	+	−	−	−	−	−	−	−	−
Arginine	+	+	−	+	−	+	−	−	−	−	−	−	+	−
Lysine	+	−	−	+	−	+	−	−	−	−	−	−	−	−
Other														
DNase[c]	+	−	+	−	−	−	+	−	+	+	+	+	+	+
Motility[c]	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] A, Acid production; N, neutral (nonreactive); B, basic reaction.
[b] Acid fast.
[c] Conventional media were used.

media. The breakdown of the 37 isolates according to the schema proposed by Weaver is outlined in Fig. 2.

Once the isolates were separated into five groups in this general plan, each group was examined for more specific metabolic reactions by using various substrates in O/F base medium. The first two isolates examined (Fig. 2, group A) were glucose oxidizers that grew on MacConkey agar and were oxidase positive. No oxidation was observed at 35°C; at 30°C, however, glucose, mannitol, sucrose, and maltose were oxidized (Table 1). For all other biochemical reactions, no growth was observed when conventional or commercially prepared media were used for these two isolates. The base media (essentially O/F base without the indicator but containing specific substrates) supported growth of the isolates; this in turn allowed expression of nitrate and nitrite reduction, decarboxylation, and dihydrolation as well as motility determination (Table 2). Additional biochemical tests selected from *Methods for Numerical Taxonomy* (3) are included here and in the remaining tables as complementary reactions.

Biochemical reactions for the five isolates that oxidized glucose and were MacConkey negative and oxidase positive are listed in Table 3. These organisms fit the Weaver plan (12) for group B members. Reactions for these were obtained with the use of the O/F base medium and a lower (30°C) incubation temperature. All five of the yellow-pigmented organisms oxidized xylose and lacked motility. The majority of the isolates gave alkaline reactions on mannitol, lactose, sucrose, and maltose. Reduction, decarboxylation, and dihydrolation reactions varied among the isolates.

Table 4 lists the four isolates that did not utilize glucose even at the lower temperature of 30°C. These organisms grew on commercially prepared MacConkey agar (Difco), were oxidase positive, and fit the Weaver plan (12) for group C. The four isolates, with few exceptions, showed consistent reduction and hydrolysis patterns. They hydrolyzed hippurate and failed to hydrolyze the other substrates. No definite pattern was observed for the amino acids. They gave negative reactions for indole and DNase and were motile.

The 12 isolates of group D (Table 5) were characterized with the use of the modified medium containing the specific substrates and incubation at 30°C. Two of the isolates were gram-negative diplococci, which were nonreactive with the API-20E system and showed a distinct colonial morphology (tiny blue) on eosin methylene blue agar (11). A DNA transformation was completed to a competent auxotroph of *A. calcoaceticus* (2, 7). This reaction enabled positive identification of the two isolates (no. 22 and 23) as *A. calcoaceticus*. Although *A. calcoaceticus* ordinarily subcultures to SPCA, neither isolate in question subcultured to SPCA (nor API-20E), but both were recovered on R3A medium. Use of the modified base medium and lower incubation temperature allowed for their biochemical characterization. The remaining 10 nonmotile gram-negative organisms showed inconsistent biochemical patterns.

Table 6 lists the 14 isolates of group E that were glucose nonoxidizers, MacConkey negative, and oxidase positive. An acid-fast organism, no. 9, was recovered by this technique. No consistent patterns of the biochemical reactions were observed.

DISCUSSION

Bacteria that were recovered from drinking water and that could not be identified because they failed to grow in most commercially prepared media grew on R3A medium; they had been lost when subcultured to SPCA. Growth or lack of growth on MacConkey agar, along with the oxidase reaction and ability to utilize glucose, permitted taxonomic separation by the plan of Weaver (12). Growth in O/F base medium, modified by addition of specific substrates and incubation at 30°C rather than at 35°C, allowed further expression of their metabolic characteristics.

These reactions thus establish a groundwork for the taxonomic characterization of these dysgonic isolates from drinking water samples. The findings emphasize the variety of metabolic types of heterotrophic bacteria encountered in drinking water. Identification of these organisms is a tedious task; the value of the results must be weighed against the fact that any potential risk posed by their presence in potable water is unknown at present.

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